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Resistance and Biofilm Production Profile of Potential Pathogens Isolated from *kpètè kpètè* Used to Produce Traditional Fermented Beer in Benin

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Abstract: This study aimed to determine the pathogenicity of the bacteria isolated and characterized from the *kpètè kpètè* used to produce two fermented beers in Benin. Species were identified by specific biochemical tests such as catalase, coagulase, and API 20 E. Antibiotic sensitivity was tested according to the French Society of Microbiology Antibiogram Committee. The crystal violet microplate technique and conventional PCR evaluated biofilm production to identify genes encoding virulence and macrolide resistance. Our data shows that *Kpètè Kpètè* used to produce beers are contaminated by *Enterobacteriaceae* species (*Klebsiella terrigena, Enterobacter aerogens, Providencia rettgeri, Chryseomonas luteola, Serratia rubidae,* and *Enterobacter cloacae*) and *Staphylococcus* spp. These multidrug-resistant strains can produce biofilms with a strong predominance of *Enterobacter aerogens, Klebsiella terrigena* (100%), and *Staphylococcus* spp (60%). *Enterobacter cloacae* (4%) and Coagulase negative *Staphylococcus* (5.55%) harbor the macrolide resistance gene. For other strains, these genes were not detected. Foods contaminated with bacteria resistant to antibiotics and carrying a virulence gene could constitute a potential public health problem. There is a need to increase awareness campaigns on hygiene rules in preparing and selling these traditional beers.

Keywords: *Kpètè Kpètè;* microbiological contaminant; Enterobacteria; resistance genes; *Staphylococcus* spp.

1. Introduction

Tchoukoutou and Tchapalo are two traditional beers sold as street food throughout Benin and prepared, respectively, from red or brown sorghum (*Sorghum bicolor*) and maize [1,2]. Aside from their social aspect, since they are used during reunions and traditional ceremonies [3], selling these beers constitutes an essential source of income, especially for women [1,4]. However, during its cooking and sale, a lack of qualitative hygiene conditions of the environment and utensils was reported. Thus, sold as street food, these drinks can cause many cases of food poisoning [5].

Food poisoning causes more than 200 illnesses, ranging from diarrhea to cancer, with nearly 420,000 deaths yearly worldwide [6]. During beer manufacturing, microorganism fermentation, one of the essential stages in the production of traditional beers, abounds in diverse microbial communities that strongly influence the sensory quality, the availability of nutrients, and the safety and preservation of these products [7–9]. In this microbial community, bacteria, yeasts, and molds come from raw materials, utensils, or the producers themselves [10,11].

Even though lactic acid bacteria reduce the level of pathogenic bacteria present in these drinks [7,8,12,13], it is still observed that resistance of certain bacteria such as *Enterobacter sakazaki*, *Klebsiella pneumonia*, *Escherichia coli*, and *Staphylococcus aureus* to the acidity of the fermented medium and the

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cooking of fermented foods [14]. Indeed N'tcha et al. [15] observed the presence of *Enterobacteriaceae* in Tchoukoutou. This presence of beer is worrying because it can cause food spoilage [16,17] and affect patients with health disorders or habitat disorders such as sepsis, meningitis, endocarditis, peritonitis, or heart disease [18].

In West African countries, bacterial infections' endemicity has increased with antibiotics consumption [19,20]. Due to the misuse and often uncontrolled use of synthetic products, controlling bacterial and fungal infections becomes complex due to the emergence of bacteria and fungi resistant to many conventional antibiotics and antifungals [21,22]. This multidrug resistance can be explained by chromosomal mutations of these strains or by acquiring resistance genes [23]. However, the literature has ignored the species of Enterobacteriaceae and most of the pathogenic bacteria identified in these traditional beers through biochemical and molecular characterization.

Indeed, the lack of hygiene during beer production and the environmental conditions during the sale, accompanied by the poor hygienic quality of the utensils used, can contribute to beer contamination by the pathogenic microorganisms responsible for food poisoning. Thus, the present study aims to characterize the pathogenic bacteria isolated from two traditional beers produced and consumed in Benin.

2. Materials and Methods

2.1. Sampling

Thirty-seven (37) isolates of enterobacteria and staphylococci used were isolated from samples of *kpètè-kpètè* of two traditional beers (Tchoukoutou and Tchapalo) collected by N'tcha et al. [15] in areas with high production of conventional beers in Benin such as Natitingou, Tanguiéta, Boukoumbé, Parakou, N'Dali, Tchaourou, Bantè, Glazoué, and Dassa Zoumè.

2.2. Microbiological analyses

2.2.1. Identification of bacterial strains

Once in the laboratory, 5 ml of Mueller Hinton broth was added to each case. The cases were then incubated for 24 hours at 37°C. After this incubation period, cloudy points indicating bacterial growth were chosen for germ tests. Isolation and purification of strains were carried out using selective media. Mac Conkey, EMB, and SS Agar media were used to isolate *Enterobacteriaceae*, and Baird Parker medium supplied with egg yolk+ potassium tellurite + 0.2% sulfamethazine and coagulase test was used for the staphylococci. Isolation was performed by subculturing the isolated colony to obtain a pure culture. The pure strains were obtained after three successive subcultures were used for biochemical and molecular identification. Phonotypic identification of isolated *Enterobacteriaceae* was completed by plating with API 20 E-commercial kit (Biomerieux, Marcy l'Etoile, France).

2.2.2. Biofilm formation

The capacity to produce biofilm of the isolates was determined [24]. Briefly, 10 µl of 18 hours old isolates suspension was diluted with 150 µl of Brain Heath Infusion before incubating for 24 hours at 37°C. After incubation, the wells were washed three times with about 0.2 ml of sterile physiological water (0.9% NaCl). Biofilms formed by the adhesion of sessile organisms to the microplate in each well were stained with crystal violet (0.1%) for 10 minutes. The violet staining in a well indicates a positive test. The excess dye was removed by thorough washing, and the plates were left at room temperature for drying [25].

2.2.3. Antibiotic susceptibility of isolates

The disc diffusion method was used to evaluate the antibiotic susceptibility of the isolated strains according to the standards and recommendations of the Antibiogram Committee of the

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French Society of Microbiology [26]. For the enterobacteria, the used antibiotics agents (Oxoid®) were ampicillin (A, 10 µg), ceftriaxone (Cl, 30 µg), cefoxitin (FOX 30 µg), cefotaxime (CTX 30 µg), chloramphenicol (C 30), gentamicin (G 10 µg), doxycycline (DO 30 µg), trimethoprim-sulfamethoxazole (STX 25 µg) and nalidixic acid (NA 30 µg). The antibiotics agents (Oxoid®) used for staphylococci were: penicillin G (1 µg), ceftriaxone (Cl 30 µg), cefotaxime (CTX 30 µg), cephalothin (KF 30 µg), vancomycin (VA 5µg), chloramphenicol (C 30), gentamicin (G 10 µg), streptomycin (S 10 µg), erythromycin (E 15 µg), trimethoprim-sulfamethoxazole (STX 25 µg) and nalidixic acid (NA 30 µg).

2.3. Molecular characterization of isolates

2.3.1. DNA extraction

DNA extraction was performed according to an adaption of the previously described method by Rasmussen et Morrissey [27]. Thus, from a fresh bacterial culture (about 18 hours old), 3 to 4 colonies were used for preculture in 1 ml of brain-heart infusion before incubation (37°C for 18 hours). The tubes were then centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, and 500 µl of TBE 1x was added to the bacterial pellet and then mixed and heated in a dry bath at 95°C for 15 minutes. After heating, the mixture was centrifuged at 12000 rpm for 5 minutes. The supernatant was recovered into a sterile tube and 500 µl of ethanol before another centrifugation at 12,000 rpm for 5 minutes. The DNA pellets were suspended in 50 µl of clean distilled water and stored at + 4°C for imminent use or at -20°C for long-term storage.

2.3.2. Search for alleles of genes encoding virulence

For *Enterobacteriaceae*, genes encoding for the synthesis of adhesins (*fimA: fim* AF*:* 5′-CGGCTC TGTCCCTSAGT-3′ and *fim* AR: 5′-GTCGCATCCGCATTAGC-3′) [28,29] and the one encoding for the synthesis of cytotoxic necrosis factor (*cnf1*: *cnf1F: 5′*-GGGGGAAGTACAG AAGAATTA-3′ and *cnf1R:* 5′-TTGCCGTCCACTCTCACCAGT-3′) [30,31]. The PCR reactions were performed using the thermal cycler using the following program: a cycle of initial denaturation (94°C/3 minutes), followed by 30 cycles of denaturation (94°C/1 minute), hybridization (52°C/1 minute), and elongation (72°C/1 minute); followed by a final extension (72°C/10 minutes).

Concerning the *Staphylococcus* spp isolates, gene alleles encoding macrolide resistance (*erm*B *ermB1: 5′*-FGAAAAGGTACTCAACCAAATA-3′ *and ermB2:* 5′-AGTAACGGTAC TTAAATTGTTTAC-3′) [32] and gene alleles encoding erythromycin resistance (*mef*A: *mef(A/E)1:* 5′-AGTATCATTAATCACTAGTGC-3′ *and mef(A/E)*2*:* 5′-TTCTTCTGGTAC TAAAAGTGG-3′) [32] were investigated. In this case, amplification conditions were: initial denaturation (95°C/3 minutes), followed by 35 cycles of denaturation (95°C/1 minute), hybridization (57°C/1 minute), and elongation (72°C/1 minute), followed by a final extension (72°C for 10 minutes).

The PCR reaction was performed on 25 µl containing 20 µl 10x GoTaq mix (PROMEGA, Madison, WI USA); 1 µl primer F (10 µM); 1 µl primer R (10 µM), and 3 µl DNA. The amplification program comprises an initial denaturation (94°C for 2 minutes).

2.4. Data analysis and processing

The results of all experiments carried out in duplicate were plotted on a bench sheet and then entered into the Excel 2016 spreadsheet. The Excel API 20 E spreadsheet was used to identify the biochemical profiles of the strains. The data were subjected to an analysis of variance (ANOVA) using the GraphPad Prism 8 software. Tukey's multiple comparisons test is used to compare the difference in means. The P value $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of pathogenic bacteria isolated

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Ninety-four bacteria strains containing *Enterobacteriaceae* (60.64%) and staphylococci (39.36%) were isolated. Of the isolated bacteria, 28.43% were coagulase-negative staphylococci (CNS), and 10.93% coagulase-positive staphylococci (CPS). Concerning the *Enterobacteriaceae*, six different species such as *Klebsiella terrigena* (1.38%), *Enterobacter aerogens* (4.14%), *Providencia rettgeri* (5.51%), *Chryseomonas luteola* (6.89%), *Serratia rubidae* (15.16%) and *Enterobacter cloacae* (27.56%) were isolated. However, the most predominant species remain CNS and *E. cloacae* (Figure 1).

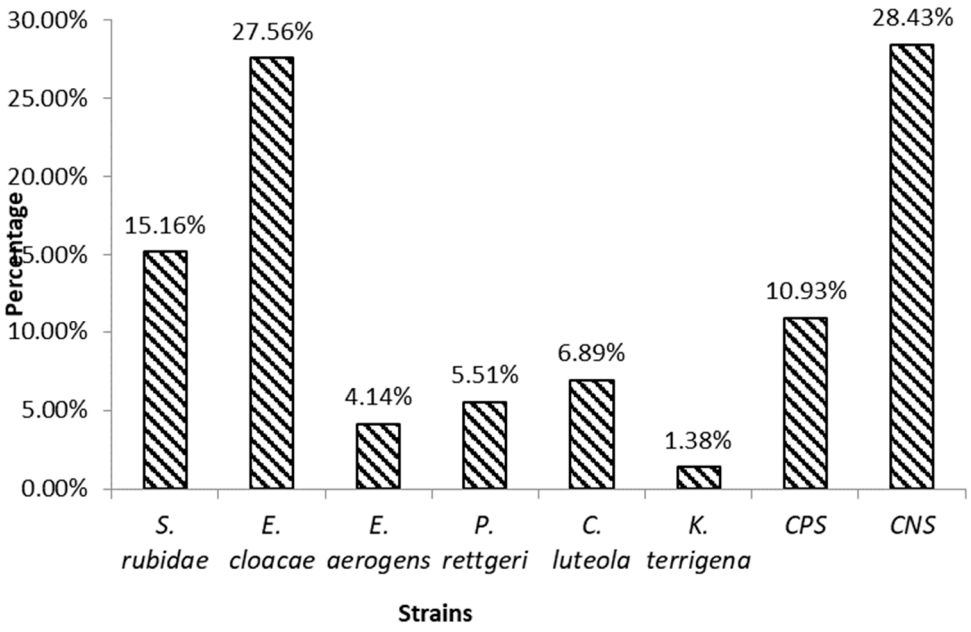

Figure 1. Distribution of *Enterobacteriaceae* strains according to species.

3.2. Biofilm production

Among the isolated Enterobacteriaceae, 44% were biofilm producers. The biofilm production rate significantly varies ($P < 0.0001$) according to the species (Figure 2). Thus, figure 2 shows that 100% *of Enterobacter aerogens* and *Klebsiella terrigena* were biofilms producers, following *Providencia rettgeri* (75%). The lowest production rate was observed with *Serratia rubidaea* (14.28%). Concerning the staphylococci, 28% were biofilm producers. As shown in Figure 2, this production level was high among coagulase-positive staphylococci (60%) compared to coagulase-negative staphylococci (15.38%).

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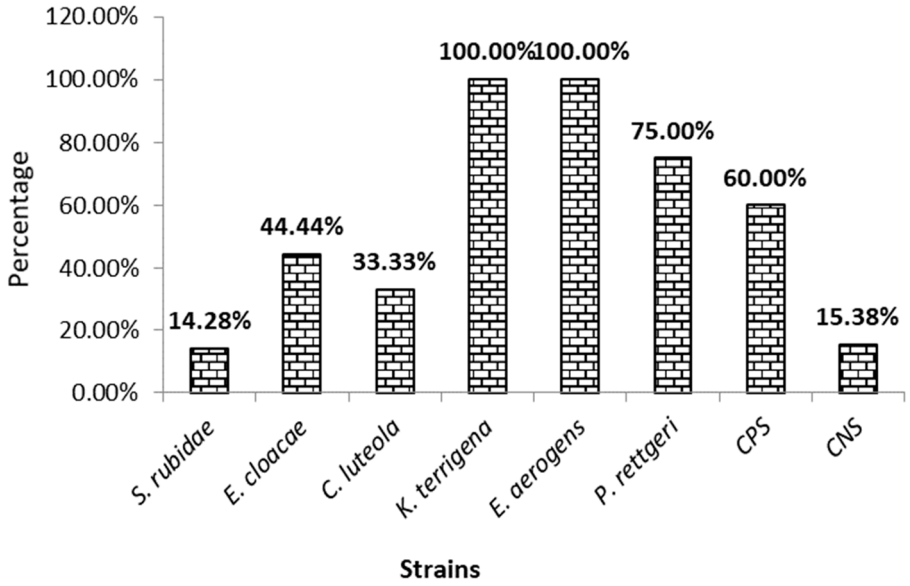

Figure 2. Distribution of biofilm production capacity according to identified species. CPS: coagulase-positive staphylococci, CNS: coagulase-negative staphylococci.

3.3. Resistance profile of Enterobacteriaceae to antibiotics by species

The isolated Enterobacteriaceae were divided into 6 species to evaluate their antibiotic susceptibility. Thus, Table 2 shows that Serratia rubidae, Enterobacter cloacae, Enterobacter aerogens, Providencia rettgeri, Chryseomonas luteola, and Klebsiella terrigena were highly resistant (100%) ampicillin, chloramphenicol, cefoxitin, and ceftriaxone. These strains showed sensitivity to gentamicin (1.85%), doxycycline (32.4%), trimethoprim/sulfamethoxazole (32.87%), and nalidixic acid (43.51%). Nevertheless, Klebsiella terrigena and Enterobacter aerogens were 100% resistant to quinolones like nalidixic acid and developed, respectively, highly immune to cyclones like doxycycline and sulfonamides like trimethoprim-sulfamethoxazole. The statistical analysis of Table 2 shows a significant difference between the effect of using antibiotics ($P < 0.001$).

Table 2. Distribution of *Enterobacteriaceae* resistance phenotypes to antibiotics.

Enterobacteriaceae		P value	
Antibiotics	**Percentage of resistance**		
Ampicillin (A10)	100.00%	**>0.9999**	**< 0.0001**
Ceftriaxone (Cl30)	100.00%		
Cefoxitin (FOX30)	100.00%		
Chloramphenicol (C30)	100.00%		
Gentamicin (G10)	1.85%	**>0.9999**	
Doxycycline (DO30)	32.40%		
Trimethoprim-sulfamethoxazole (STX25)	32.85%		
Nalidixic acid (NA30)	43.51%		

Gentamicin completely inhibited all strains of *Enterobacteriaceae* without *Enterobacter cloacae,* but only 11.11 % of *Enterobacter cloac*ae showed resistance to this antibiotic. Figure 3 shows that only *Enterobacter cloacae* developed resistance to all-over antibiotics, followed by *Providencia rettgeri,* while *Chryseomonas luteola* remained the most sensitive strain.

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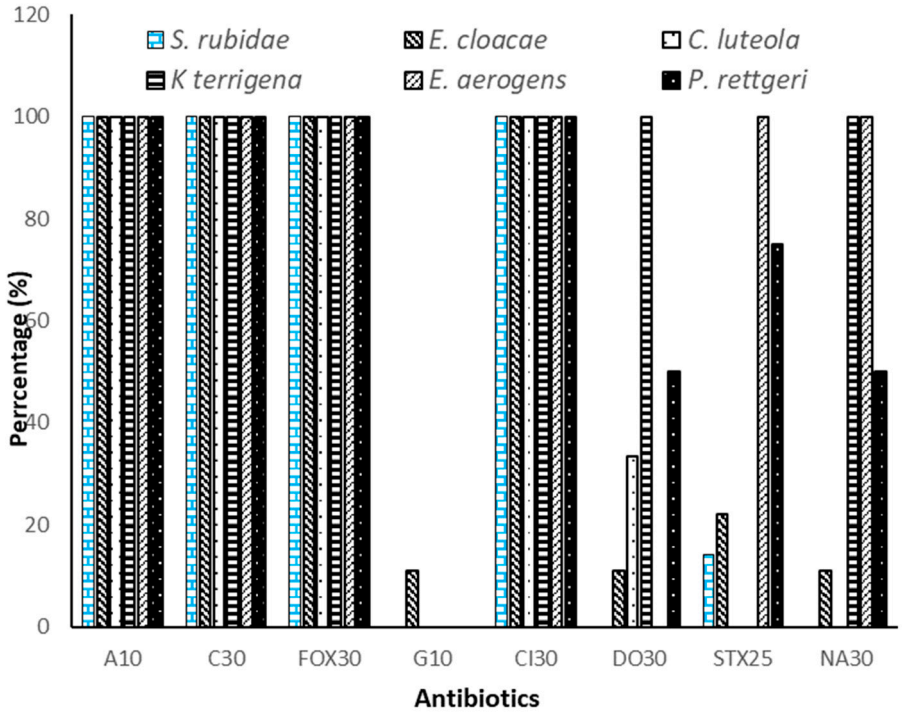

Figure 3. Resistance profile of isolated Enterobacteriaceae to tested antibiotics. A10: Ampicillin (10µg), C30: Chloramphenicol (30 µg), FOX30: Cefoxitin (30 µg), G10: Gentamicin (10µg), Cl30: ceftriaxone (30 µg), DO30: Doxycycline (30µg), SXT25: trimethoprim -sulfamethoxazole (25 µg), NA30: nalidixic acid (30 µg).

3.4. Resistance profile of Enterobacteriaceae to antibiotics by species

Staphylococci have been divided into two categories for assessing susceptibility to antibiotics: *Staphylococcus aureus* (CPS) and *Staphylococcus spp* (CNS). Figure 4 shows the results of the antibiotic resistance profile of staphylococci.

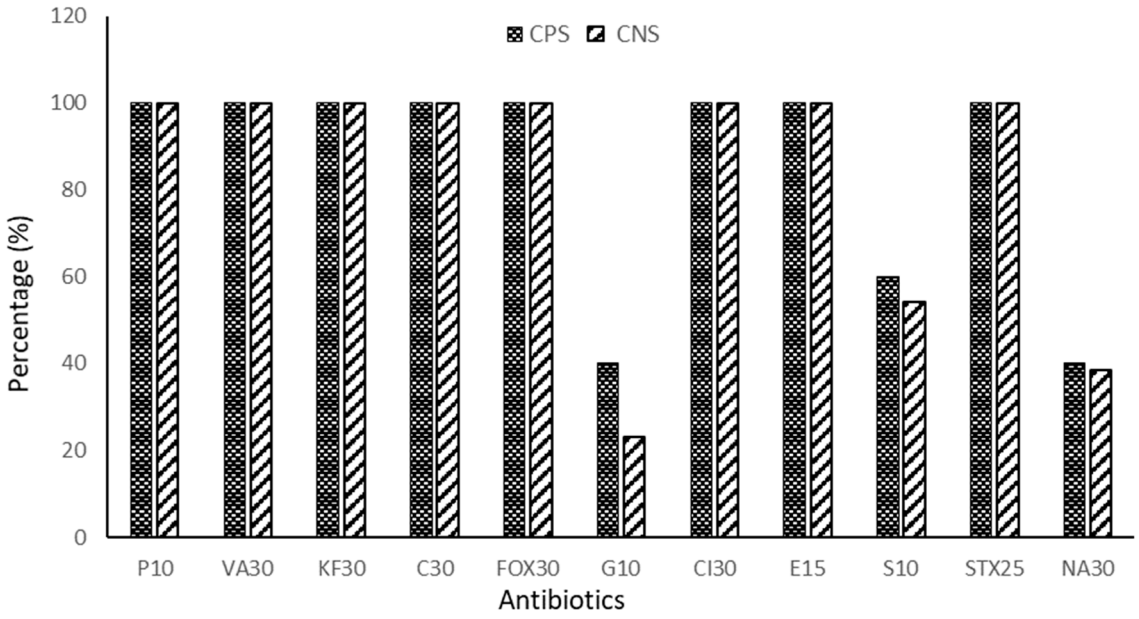

Figure 4. Resistance profile of staphylococci to tested antibiotics. CPS: Staphylococcus aureus, CNS: Staphylococcus spp, P 10: Penicillin (10µg), VA30: Vancomycin (30µg), KF 30: Cephalotin (30µg), C30: Chloramphenicol (30 µg), FOX: Cefoxitine (30 µg), G10: Gentamicin (10µg), Cl30: ceftriaxone (30 µg),

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E15: Erythromycin(15µg), S10: Streptomycin (10µg), SXT2: trimethoprim-sulfamethoxazole (25 µg), NA30: Nalidixic acid (30 µg).

The statistical analysis of Table 3 shows that there is a significant difference between the use of antibiotics ($P < 0.001$). Table 3 shows that all strains of staphylococci are 100% resistant to β-lactams, particularly penicillin G, cefoxitin, cephalothin, chloramphenicol, and ceftriaxone, sulfonamides such as trimethoprim-sulfamethoxazole, glycopeptides such as vancomycin and macrolides like erythromycin. However, their resistance was low to gentamicin (31.54%), nalidixic acid (39.23%), and streptomycin (59.92%). Gentamicin shows low efficacy against staphylococci, particularly *Staphylococcus aureus.*

Table 3. Distribution of antibiotics resistance phenotypes of staphylococci.

Staphylococci		**P value**	
Antibiotics	**Percentage of resistance**		
Penicillin G (P10)	100.00%		
Ceftriaxone (Cl30)	100.00%		
Cefoxitin (FOX30)	100.00%		
Chloramphenicol (C30)	100.00%		
Cephalothin (KF30)	100.00%	**>0.9999**	
Vancomycin (VA30)	100.00%		**<0.0001**
Erythromycin (E15)	100.00%		
Trimethoprim-sulfamethoxazole (STX25)	100.00%		
Gentamicin (G10)	31.54%		
Nalidixic Acid (NA30)	39.23%	**>0.9999**	
Streptomycin (S10)	59.92%		

3.5. Virulence genes and Resistance gene macrolides

In this study, 4% of *Enterobacteriaceae* strains harbored *the fimA* gene, and 5.55% of staphylococci strains contained *the ermB* gene encoding macrolide resistance. However, there is no *cnf1* and *mefA* gene for *Enterobacteriaceae* exhibiting enterotoxin produced by *E. coli* and staphylococci showing erythromycin resistance.

4. Discussion

The primary source of human infections is ingesting contaminated food [33]. Indeed, pathogenic bacteria such as staphylococci and *Enterobacteriaceae* have been identified in African fermented cereal foods [34] and traditional fermented drinks [15]. Our study showed that the percentages of samples contaminated with these microorganisms were 60.64% of *Enterobacteriaceae* and 39.36% of staphylococci. This rate of *Enterobacteriaceae* contamination is much higher than the 14.4% obtained by Cason et al. [35] in Sesotho, a traditional beer from South Africa produced from corn or sorghum. The low rate of *Enterobacteriaceae* and the absence of staphylococci in Sesotho can be explained by the fact that Sesotho is obtained from two fermentations with the addition of three different ferments (Tomoso, Mmela, and Yomoso) and by the fact that the two studies were not carried out under the same conditions. However, observing the rules of Good Hygiene Practices and Good Preparation Practices can reduce contamination rates in this situation.

The biochemical characterization of the enterobacteria and staphylococci isolated strains gave us more precision on the species in these traditional beers. We noted the presence of *Klebsiella terrigena* (2.28%), *Enterobacter aerogens* (6.82%), *Providencia rettgeri* (9.09%), *Chryseomonas luteola* (11.36%), *Serratia rubidae* (25%) and *Enterobacter cloacae* (45.45%) for *Enterobacteriaceae*. As for the *Staphylococcus* genus, we noted the presence of 13.51% of coagulase-positive staphylococci (probably *Staphylococcus aureus*) and 86.48% of coagulase-negative staphylococci (*Staphylococcus* spp). Certain pathogenic

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microorganisms and alterations of traditional beers observed in Sesotho, namely, the genus *Chryseobacterium*, *Enterobacter,* and *Klebsiella* [35], were also present in our samples. These strains have also been isolated in other traditional beers prepared from raw materials, such as rice for Chicha, a Brazilian fermented drink [36], and cactus juice for pulque, a Mexican alcoholic beverage [37]. The high proportion of *Enterobacter cloacae* (45.45%) observed in these traditional beers could be due to its habitat, which remains the environment. The results of our study corroborate those of several studies [35–37] by highlighting the presence of unusual species such as *Serratia rubidae*, *Providencia rettgeri*, and *Staphylococcus aureus* in our samples. Indeed, *Serratia rubidae* and *Providencia rettgeri* have been found in stool [38] and fly [39], respectively. *Staphylococcus* are ubiquitous bacteria ubiquitous in nature, i.e., air, water, soil, food, furniture, and equipment [18,40]. The presence of these different species can be explained by their contamination of the beer production process, raw materials, the sales environment, lack of supervision, or non-compliance with the hygiene rules.

During bacterial infections, the way of life developed by bacteria is biofilm production [41]. The production of biofilm is one of the defense mechanisms of bacteria. Biofilm formation is a default lifestyle for staphylococci and *Enterobacteriaceae* [42,43]. The high rate of biofilm production observed in *Enterobacter aerogens* and *Klebsiella terrigena* during our research can be explained by the fact that they are Gram-negative thermotolerant bacilli of food origin. Regarding staphylococci, the low rate observed in Coagulase Negative Staphylococci (15.35%) was also found in the work of Ahouandjinou et al. [44], with a respective proportion of biofilm production of 28% for *Staphylococcus lugdunensis* and 20% *S. warneri* on food Coagulase Negative Staphylococcus (CNS) strains. Indeed, some CNSs, such as *Staphylococcus epidermidis*, adhere more quickly to the stainless steels of food industry equipment [45]. Therefore, these CNSs can easily comply with the kitchen utensils involved in producing these traditional beers. Therefore, properly cleaning these equipment and kitchen utensils could justify the low rate observed in the CNS. As for *S. aureus* (60%), the high rate observed can be explained by the fact that they adhere and quickly develop biofilm in contact with the food surface [46]. This lifestyle facilitates the adhesion of bacteria to food surfaces creating a public health problem [47]. In short, in these beers, the high rate of contamination observed with *Providencia rettgeri*, *Enterobacter aerogens*, *Klebsiella terrigena,* and *Staphylococcus aureus* can be explained by the fact that they can produce biofilm, which provides specific adhesion characteristics allowing their persistence and their adaptation to bad conditions. The production of biofilm is potentially a risk of poisoning for the consumer.

Gentamicin and nalidixic acid had shown efficacy in almost all strains isolated except *Enterobacter cloac*ae. Our results corroborate those of Hama et al. [48] and Anago et al. [49], who qualify gentamicin as having excellent efficacy against staphylococci and ESBL-producing strains of enterobacteria, respectively. This effectiveness of gentamicin, accompanied by nalidixic acid, can be explained by the fact that aminoglycosides (gentamicin) act on the bacterial ribosome and induce the synthesis of erroneous proteins [50]. As for quinolones (nalidixic acid), they exert their action at the time of DNA replication by acting on DNA gyrase and topoisomerase IV, which regulate the topology of DNA to allow replication [51]. The inhibition of DNA gyrase leads to the suppression of the positive supertowers of DNA.

In contrast, that of topoisomerase IV leads to the accumulation of daughter replicons, thus disrupting this replication. In addition to gentamicin and nalidixic acid, we note that some antibiotics, such as doxycycline, trimethoprim-sulfamethoxazole, remain active on enterobacteria and streptomycin on staphylococci. A study conducted by Kadja et al. [52] on combining tetracycline and trimethoprim sulfonylurea on strains from dairy farming corroborated our results with an efficacy of 62% and 67%, respectively. As for β-lactams and others, the *Enterobacteriaceae* were all resistant (100%). Previous work corroborates our results on the sensitivity of *Enterobacteriaceae* strains isolated to the antibiotics tested [53,54]. In addition, some work has shown that vancomycin, penicillin G, and macrolides are effective against food-borne *S. aureus* strains [52,55]. Globally, the resistance of *Enterobacteriaceae* and staphylococci isolates to β-lactams can be explained by the production of β-lactamase, which hydrolyzes these antibiotics or the reduction of the structure of the pores by the antibiotics which pass [56]. The development of resistance to other antibiotics can be explained by

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the fact that these strains have already been in contact with different families of antibiotics. Particularly for staphylococci, resistance to ceftriaxone implies resistance to almost all β-lactams currently available [57] and the development of resistance to many antibiotics widely used to control infections such as food poisoning [58,59]. The multidrug resistance of the isolates could be explained by self-medication and excessive and uncontrolled use of antibiotics accompanied by a selection of resistant bacteria [60,61]. This selection could be due to acquiring resistance genes such as erm, *msr*, *mef*, and *mph* [56] or virulence genes such as *fimA* and *cnf1* [62].

Our study only revealed a shallow presence of *fimA* and ermB genes in *Enterobacteriaceae* (*Enterobacter cloacae*) and staphylococci (CNS). The work of Le Trong [63] showed the *fimA* gene's presence in many *Enterobacteriaceae* species. In 2020, Soria-Bustos et al. [64] isolated *Enterobacter cloac*ae with virulence properties in the appearance of extra-intestinal infections. Thus, the biofilm production capacity of *E. cloac*ae could be explained by the fimA gene, which codes for the synthesis of adhesin, an essential protein in biofilm formation. As for the cnf1 gene, its absence can be explained by the fact that it is mainly produced by *E. coli* [65]. Regarding staphylococci, a study on the transferability of antibiotic resistance genes between bacteria carried out during food fermentation of milk showed that *Staphylococcus aureus* and potentially pathogenic coagulase-negative staphylococci are carriers of macrolide resistance genes [66,67].

However, the low proportion of *ermB* genes with a complete absence of *mefA* genes, even though staphylococci were phenotypically resistant to erythromycin in our study, can be explained by the fact that these staphylococci could develop other resistance mechanisms through the acquisition of other genes such as *msr*, *mph* and *ere*, encoding for macrolides resistance. Apart from the addition of genes, the multidrug resistance of strains can be explained by forming a biofilm that protects bacteria from antibacterial molecules [68]. There is a correlation between the ability to form biofilms and antibiotic resistance. This antibiotic resistance can be explained by the diffusion of drugs in the biofilm [69]. After exposure to these agents, a small surviving population can repopulate the surface immediately and become more resistant to antimicrobial treatment. Food contaminated with bacteria resistant to antibiotics and carrying genes for resistance or virulence are dangerous germs that could threaten public health.

5. Conclusions

From our research, it is found that the *kpètè kpètè* used to produce traditional fermented beers (Tchoukoutou and Tchapalo) are contaminated with pathogenic bacteria like *Serratia rubidae*, *Enterobacter cloacae*, *Enterobacter aerogens*, *Providencia rettgeri*, *Chryseomonas luteola*, *Klebsiella terrigena*, and *Staphylococcus aureus*. These bacteria exhibit multidrug resistance to antibiotics with the ability to form biofilms. The presence of these strains in traditional drinks is a reality and remains worrying for the consumer's health. It would be better to extend this study to other types of fermented foods, to deepen this study on the search for bacterial toxins and genes for antibiotic resistance given the phenotypic character resistant to macrolides in general and erythromycin in particular that we observed in this study.

Author Contributions: Conceptualization, NC, HS, and L.B-M.; methodology, BB, RA, and DNB; software, DNB and RA; validation, NC, HS, AA, IMSH, and L.B-M.; formal analysis, DNB, IMSH, and HS; investigation, NC and BB; resources, OOB and L.B-M.; data curation, NC, BB, and HS; writing—original draft preparation, NC and DNB; writing—review and editing, JF-M; OOB and L.B-M.; visualization, HS; supervision, JF-M; OOB and L.B-M..; project administration, AA. HS and LB-M.; funding acquisition, OOB; AA; HS All authors have read and agreed to the published version of the manuscript.

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References

1. ayodé, APP; Linmemann, A.R.; Nout, M.J.R.; Hounhouigan, D.J.; Stomph, T.J.; Smulders, M.J.M.; "Diversity and food quality properties of farmers' varieties of sorghum from Benin," *Journal of Science of Food and Agriculture*, **2006**, vol. *86, pp. 1032–1039*.
2. Missihoun, A.A. ; Agbangla, C. ; Sagbadja, H. A. ; Ahanhanzo, C. ; Vodouhe, R. ; "Gestion traditionnelle et statut des ressources génétiques du sorgho (*Sorghum bicolor* L. Moench) au Nord-Ouest du Bénin," *International Journal of Biological and Chemical Sciences*, **2012**, *vol. 6, no. 3, pp. 1003-1018.*
3. Aholou, C. C. "Construction identitaire et urbaine autour du cabaret à Lomé (Togo)," *Hommes et migrations*, 2010, *vol. 1283, pp. 32-41 https://doi.org/10.4000/hommesmigrations.985*
4. Osseyi, E. G., Tagba, P.; Karou, S. D. "Stabilization of the Traditional Sorghum Beer "Tchoukoutou" using Rustic Wine- Making Method," *Advance Journal of Food Science and Technology*, 2011, *vol. 3, no. 4, pp. 254-258*.
5. Baba-Moussa, L. ; Bokossa, Y. I. ; Baba-Moussa, F. ; Ahisou, H. ; Adeoti, Z. ; Yehouenou, B. ; Mamadou, A. Toukourou, F. ; Sanni, A. "Etude des possibilités de contamination des aliments de rues au Bénin : Cas de la ville de Cotonou," *Journal de Recherche Scientifique de l'Université de Lomé (Togo) série A, 2006, vol. 8, no. 2, pp. 149-156*.
6. WHO. Rapport OMS : 420 000 personnes tuées par l'intoxication alimentaire. 2018.
7. Adesetan, T. O., Efuntoye, M. O., Babalola, O. O. "Profiling of Bacillus Cereus Enterotoxigenic Genes from Retailed Foods and Detection of the Nhe and Hbl Toxins with Immunological Assay". *Journal of Applied and Natural Science*, 2022, 14, 254-267 DOI:https://doi.org/10.31018/jans.v14i1.3258.
8. Imade, E.E., Omonigho, S.E., Babalola, O.O., Enagbonma, B.J. "Lactic acid bacterial bacteriocins and their bioactive properties against food-associated antibiotic-resistant bacteria." *Annals of Microbiology*, 2021, vol. 71, n°44. https://doi.org/10.1186/s13213-021-01652-6
9. Chuah, L.O.; Shamila-Syuhada, A.K.; Liong, MT; Rosma, A.; Thong, K. L.; Rusul, G. "Physio-chemical, microbiological properties of tempoyak and molecular characterisation of lactic acid bacteria isolated from tempoyak," *Food Microbiology,2016, vol.* 58, pp. 95–104.
10. Egbuta, M. A., Mwanza, M., Babalola, O. O. "Health Risks Associated with Exposure to Filamentous Fungi." International Journal of environmental research and public health, 2017, vol. 14, n°7, 719. https://doi.org/10.3390/ijerph14070719
11. Achi, O. K.; Ukwuru, M. "Cereal-based fermented foods of Africa as functional foods," *International Journal of Microbiology Applied, 2015, vol.* 2, pp. 71–83.
12. Adesetan, O., T., Efuntoye, M. O., Babalola, O. O. "Biochemical Characterization and Antimicrobial Susceptibility of Bacillus Cereus Isolates from Some Retailed Foods in Ogun State, Nigeria. *Journal of Microbiology, Biotechnology and Food Sciences*, 2019, *9*(3), 616–621. https://doi.org/10.15414/jmbfs.2019/20.9.3.616-621
13. Kimmons, J. E.; Brown, K. H.; Lartey, A.; Collisone, M. P. P.; Dewey. K. G. "The effects of fermentation and/or vacuum flask storage on the presence of coliforms in complementary foods prepared in Ghana," *International Journal of food Sciences and Nutrition, 1999, vol. 50, pp.195-201.*
14. Akaki, K. D. ; Aw, S. ; Loiseau, G. ; Guyot, J. P. "Etude du comportement des souches de *Bacillus cereus* ATCC 9139 et d'*Escherichia coli* ATCC 25922 par la méthode des challenge-tests lors de la confection de bouillies à base de pâte de mil fermentée en provenance de Ouagadougou (Burkina-Faso)," *Revue Ivoirienne des Sciences et Technologie*, 2008, vol. 11, pp. 103-117.
15. N'tcha, C.; Adéyèmi, A. D.; Kayodé, A. P. P.; Vieira-Dalodé, G.; Agbobatinkpo, B. P.; Codjia, J. C.; Baba-Moussa, L. "Indigenous knowledge associated with the production of starters culture used to produce Beninese opaque sorghum beers," *Journal of Applied Biosciences,2015, vol. 88, pp. 8223-8234*.
16. Lyhs, U.; Björkroth, J.; Hyytiä, E.; Korkeala, H. "The spoilage flora of vacuumpackaged, sodium nitrite or potassium nitrate treated, cold-smoked rainbow trout stored at 4ºC or 8ºC," *International Journal of food microbiology,1998, vol. 45, pp. 135-142*.
17. Sundaramoorthy, N.; Yogesh, P.; Dhandapani, R.; "Production of prodigiosin from Serratia marcescens isolated from soil," *Indian Journal of Sciences and Technology*, 2009, *vol. 2, no. 10, pp. 32-34*.
18. Buchanan R. E.; Gibbons, N. E. "Bergey's Manual of Determinative Bacteriology" 8th Edition, *Baltimore, USA, Williams and Wilkcns Company*, 1974.
19. Hounsa, A.; Kouadio, L.; De Mol, P. "Self-medication with antibiotics obtained from private pharmacies in Abidjan, Ivory Coast," *Médecine et maladies infectieuses 2009, vol. 40, no. 6, pp. 333-340.*
20. Alsan, M.; Morden, N.; Gottlieb, J. D.; Weiping Z.; Skinner, J. "Antibiotic use in cold and flu season and prescribing quality: a retrospective cohort study," *Medical care,2015, vol. 53, no. 12, pp. 1066.*
21. Singh, A.; Gautam, P. K.; Verma, A.; Singh, V.; Shivapriya, P. M.; Shivalkar, S.; Sahoo, A K.; Samanta, S. K. "Green synthesis of metallic nanoparticles as effective alternatives to treat antibiotics resistant bacterial infections: A review," *Biotechnology Reports*, 2020, vol. *25*, e00427.
22. Akindolire, M. A., Babalola, O. O., Ateba, C. N. "Detection of Antibiotic Resistant Staphylococcus aureus from Milk: A Public Health Implication." *International Journal of Environmental Research and Public Health*, 2015, 12(9), 10254–10275. https://doi.org/10.3390/ijerph120910254

23. Muylaert A. ; Mainil, J. G. "Résistances aux fluoroquinolones : la situation actuelle," *Annales de Medecine Veterinaire, 2013, vol. 157, pp. 15-26*.
24. Christensen, G.D.; Simpson, W.A.; Bisno, A.L.; Beachy, EH "Adherence of biofilm producing strains of *Staphylococci epidermidis* to smooth surfaces," *Infections and Immunity,* 1985, vol. 37, pp. 318-326.
25. Stepanović, S.; Vuković, D.; Dakić, I.; Savić, B.; Švabić-Vlahović, M. "A modified microtiter-plate test for quantification of staphylococcal biofilm formation" *Journal of Microbiology Methods,2000,* vol. 40, no. 2, pp. 175-179.
26. CASFM "Comité de l'antibiogramme de la Société Française de Microbiologie : Recommandations 2020 V.1.1," 181 p, 2020. Avaible on https://www.sfm-microbiologie.org/wp-content/uploads/2020/04/CASFM2020_Avril2020_V1.1.pdf
27. Rasmussen R. S.; Morrissey, M. T. "DNA-based methods for the identification of commercial fish and seafood species." *Comprehensive Reviws in Food Science and Food Safetay*, 2008, vol. 7, no. 3, pp. 280-295.
28. Lamy, M. C.; Dramsi, S.; Billoët, A.; Réglier-Poupet, H.; Tazi, A.; Raymond, J.; Guérin, F.; Couvé, E.; Kunst F.; Glaser, P. "Rapid detection of the highly virulent group B *Straptococcus ST-17* clone," *Microbial and Infection, 2006, vol. 8, no. 7, pp. 1714-1722*.
29. Kaczmarek, A.; Budzyńska A.; Gospodarek, E. "Prevalence of genes encoding virulence factors among *Escherichia coli* wiyh K1 antigen and-K1 *E. coli* strains," *Journal of Medical Microbiology, 2012, vol. 61, no. 10, pp. 1360-1365*.
30. Moulin-Schouleur, M.; Schouleur, C.; Tailliez, P. ; Kao, M. R.; Brée, A.; Germon, P.; Oswald, E.; Mainil, J.; Blanco M.; Blanco, J. "Common Virulence factors and genetic relatonships between O18 : K1 : H7 *Escherichia coli* isolates of human and avian origin," *Journal of Clinical Microbiology, 2006, vol. 44, no. 10, pp. 3484-3492*.
31. Germon, P.; Chen, Y. H.; He, L.; Blanco, J.E.; Bree, A.; Schouler, C.; Huang, S. H.; Moulin-Schouleur, M. "IbeA, a virulence factors of avian pathogenenic *Escherichia coli*, *Microbiology, 2005, vol. 151, no. 4, pp. 1179-1186*.
32. Desjardins, M.; Delgaty, K. L.; Ramotar, K.; Toye, B. "Prevalence and mechanisms of erythromycin resistance in group A and group B *Streptococcus*: Implications for reporting susceptibility results," *Journal of Clinical Microbiology, 2004, vol. 42, no. 12, pp. 5620-5623*.
33. Griffin P.; Findlay,G. E. "Process and engineering improvements to rotating biological contactor design," *Water Sciences Technology,2000, vol. 41, no. 1, pp. 137–144.*
34. Tankoano, A. ; Diop, M. B. ; Sawadogo-Lingani, H. ; Kaoré D. ; Savadogo, A. ; "Les aspects technologiques, microbiogiques et nutritionnels de saliments fermentés à base de lait de mil en Afrique de l'ouest," *International Journal of Advanced Research, 2017, vol. 5, no. 8, pp. 1509-1526.*
35. Cason, D. E.; Mahlomaholo, J. B.; Taole, M. M.; Abong, O. G.; Vermeulen, J, G.; de Smidt, O.; Vermeulen, M.; Steyn, L.; Valverde A.; Vijoen, B. "Bacterial and Fungal Dynamics during the Fermentation Process of Sesotho, a Traditional Beer of Southern Africa," *Frontiers in microbiology,2020, vol. 11, pp. 1451.*
36. Escalante, A.; Maria, E. R.; Martinez A.; Munguia, A. L. "Characterization of bacterial diversity in Pulque, a traditional Mexican alcoholic fermented beverage, as determined by 16S rDNA analysis," *FEMS Microbiology Letters,2004, vol. 235, no. 2, pp. 273*.
37. Bokulich, N. A.; Bamforth, C. W.; Mills, D. A. "Brewhouse-resident microbiota is responsible for multi-stage fermentation of American coolshipale," *Journal Plos One,2012, 7: e35507, doi:10.1371/journal.pone.0035507.*
38. Taylor G.; Keane, P. M. "Cross-infection with *Serratia marcescens," Journal of Clinical Pathology,1962, vol. 15, pp. 145–147*.
39. Kuzina, L.; Peloquin J.; Vacek, D. "Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae)," *Current Microbiology; 2001, vol. 42, no. 4, pp. 290–294*.
40. Bensakhria. A. Biologie: *Staphylococcus aureus* (staphylocoques) , 2017 www.magazinescience.com. Accessed on 05 may 2019.
41. Kostaki, M.; Chorianopoulos, N.; Braxou, E.; Nychas G.J.; Efstathios, G. "Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions," *Applied and Environmental Microbiology*,2012, vol. 8, no. 8, pp. 2586–2595.
42. Jefferson, K. "What drives bacteria to produce a biofilm?" *FEMS microbiology letters, 2004, vol. 236, no. 2, pp. 163-173.*
43. Balestrino, D.; Ghigo, J.M.; Charbonnel, N.; Haagensen, J. A.; Forestier, C. "The characterization of functions involved in the establishment and maturation of *Klebsiella pneumoniae in vitro* biofilm reveals dual roles for surface exopolysaccharides," *Environmental Microbiology,2008, vol. 10, pp. 685–701.*
44. Ahouandjinou, H. Qualité microbiologique, antibiorésistance des souches de *Staphylococcus spp* et d'*Escherichia coli* isolées des carcasses bovines au Bénin, *PhD dissertation University of Abomey- Calavi (Benin). 2016, 175p.*

45. Lerebour, G.; Cupferman, S.; Bellon-Fontaine, M.N. "Adhesion of Staphylococcus aureus and Staphylococcus epidermidis to the Episkin reconstructed epidermis model and to an inert 304 stainless steel substrate" *Journal of Applied Microbiology*, 2004, vol. 97, no. 1, pp. 7-16.
46. Marques, S. C.; Silva-Rezende, J. G.; de Freitas-Alves, L. A. "Formation of biofilms by *Staphylococcus aureus* on stainless steel and glass surfaces and its resistance to some selected chemical sanitiziers" *Brazilian Journal of Microbiology,2007, vol. 38, no. 3, pp. 538–543.*
47. Mah, T. F. "Biofilm-specific antibiotic resistance" *Future Microbiology, 2012,vol. 7, no. 9, pp. 1061–1072.*
48. Hama H., Recherche de bactéries associées aux mammites subcliniques dans le lait de chèvre en Mauritanie et au Togo et détermination de leur antibiosensibilité. *Doctorate dissertation Veterinary Medicine, Dakar (Senegal), 2004,31.*
49. Anago, E.; Ayi-Fanou, L.; Akpovi, C. D.; Hounkpe, W. B.; Tchibozo, M.; Bankole, H. S. "Antibiotic resistance and genotype of beta-lactamase producing *Escherichia coli* in nosocomial infections in Cotonou, Benin" *Annals of Clinical Microbiology and Antimicrobials,* 2015,vol. *14, no.* 1, pp. 1-6.
50. Touati, A.; Brasme, L.; Benallaoua, S.; Gharout, A.; Madoux, J.; De Champs, C. "First report of qnrB-producing *Enterobacter cloacae* and qnrA-producing *Acinetobacter baumannii* recovered from Algerian hospitals," *Diagnostical Microbiology Infectious Diseases, 2008, vol. 60, pp. 287-290.*
51. Soussy, C. J. Quinolones et bactéries à Gram négatif. *Dans: Patrice Courvalin, Roland Leclercq, Edouard Bingen eds. Antibiogramme, 2006,2ème édition ESKA, Paris. 277p.*
52. . Kadja, M. C.; Kane, Y.; Viban, B. V.; Kaboret Y.; Alambedji, B. Sensibilité aux antibiotiques des Bactéries associées aux mammites Clinique des petits ruminants dans la région de Dakar, *Annales des Sciences Agronomique,2013, vol. 17, no. 2, pp. 205-216.*
53. Davin-Regli, A.; Bolla, J. M.; James, C. E.; Lavigne, J. P. Chevalier J.; Garnotel, E. "Membrane permeability and regulation of drug "influx and efflux" in enterobacterial pathogens," *Current drug Targets, ,2008, vol. 9, pp. 750–759.*
54. Wilson, K. Shafiee, M.; Hasso, M.; Sharif, U. "*Providencia rettgeri*: an unexpected case of Gram-negative cellulitis", *Case Report, Wounds International,2015, vol 6, no. 4.*
55. Sina, H.; Attien, P.; Wélé, M.; Socohou, A.; Boukary-Chabi, A.; Dougnon, V. T.; Baba-Moussa, F.; Adjanohoun, A.; and Baba-Moussa, L. "Sanitary Risk Factors and Microbial Contamination of Grilled Meats Sold in Cotonou, Benin", *Journal of Food Security,2019, vol. 7, no. 5, pp. 175-182.*
56. S. Schwarz, and E. Chaslus-Dancla, "Use of antimicrobials in veterinary medicine and mechanisms of resistance," *Veterinary Research, 2001, vol. 32, pp. 201-225.*
57. Chiquet, C.; Max, M.; Altayrac, J.; Aptel, F.; Boisset, S.; Vandenesch, F.; Carricajo, A. "Correlation between clinical data and antibiotic resistance in coagulase negative *Staphylococcus species* isolated from 68 patients with acute post-cataract endophthalmitis," *Clinical Microbiology and Infection, 2015,vol. 21, no. 6, 592.el-592.e8.*
58. Bonnel, A. S. ; Quinque, K. ; Le Roux, P. ; Le Luyer, B. Aspergillose nécrossante semi invasive chez un enfant de 14 ans atteint de mucoviscidose, *Revue des maladies Respiratoires,2006 vol. 23, no. 4, pp. 343-347.*
59. Rodrigues, M. X.; Silva, C. C. N.; Trevillin, H. J.; Cruzado, B. M.; Mui, S. T.; Sanches Duarte, R. F.; Castillo, C. C.; Canniatti-Brazaca G. S.; Porto, E. "Antibiotic resistance and molecular characterization of Staphylococcus species from mastitic milk" *African Journal of Microbiology Research,2017, vol. 11, no. 3, pp. 84-91.*
60. Lesch, C.; Itokazu, G.; Danziger, L.; Weinstein, R. "Multi-hospital analysis of antimicrobial usage and resistance trends," *Diagnostic microbiology and infectious disease, 2001, vol. 41, no. 3, pp. 149-154.*
61. Trivedi, K.; Cupakova S.; Karpiskova, R. "Virulence factors and antibiotic resistance in *enterococci* isolated food-stuffs," *Veterinarni Medicina, 2011, vol. 56, no. 7, pp. 352-357.*
62. Clegg S., Hughes, K. T. "FimZ is a molecular link between sticking and swimming in *Salmonella enterica serovar Typhimurium" Journal of Bacteriology,"2002, vol. 184, pp. 1209–1213.*
63. Le Trong, I. "Donor strand exchange and conformational changes during *E. coli* fimbrial formation," *Journal of Structural Biology, 2008, vol. 172, pp. 380–388.*
64. Soria-Bustos, J.; Ares, M.A.; Gómez-Aldapa, C.A.; González-y-Merchand, J.A.; Girón, J.A.; De la Cruz, M.A. "Two Type VI Secretion Systems of *Enterobacter cloacae* Are Required for Bacterial Competition, Cell Adherence, and Intestinal Colonization" *Frontier in Microbiology,2020, vol.* 11: 560488.
65. Lockman, H. A. "*Yersinia pseudotuberculosis* produces a cytotoxic necrotizing factor" *Infection and Immunity, 2002, vol. 70, pp. 2708–2714*
66. Jensen, L. B.; Frimodt-Moller N.; Aarestrup, F. M. Presence of erm gene classes in Gram-positive bacteria of animal and human origin in Denmark. *FEMS Microbiology Letters, 1999, vol. 170, pp. 151–158.*
67. Luthje, P. ; Schwarz, S. "Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide lincosamide resistance phenotypes and genotypes," *Journal of Antimicrobial Chemotherapy, 2006, vol. 57, pp. 966–969.*
68. Donlan R. M.; Costerton, J. W. "Biofilms: survival mechanisms of clinically relevant microorganisms," *Clinical Microbiology Reviews, 2002, vol. 15, pp. 167–193.*

69. Zhang, L.; Mah, T. F. "Involvement of a novel efflux system in biofilm-specific resistance to antibiotics," *Journal of bacteriology*, 2008 vol. 190, pp. 4447–4452.